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ORIGINAL ARTICLE

Potential pathogenetic role of Th17, Th0, and Th2 cells in erosive and reticular oral lichen planus

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OBJECTIVES: The role of Th17 cells and associated cytokines was investigated in oral lichen planus.
MATERIAL AND METHODS: 14 consecutive patients with oral lichen planus were investigated. For biological studies, tissues were taken from reticular or erosive lesions and from normal oral mucosa (controls) of the same patient. mRNA expression for IL-17F, IL-17A, MCP-1, IL-13, IL-2, IL-10, IL-1β, RANTES, IL-4, IL-12B, IL-8, IFN-γ, TNF-α, IL-1α, IL-18, TGF-β1, IL-23R, IL-7, IL-15, IL-6, MIG, IP-10, LTB, VEGF, IL-5, IL-27, IL-23A, GAPDH, PPIB, Foxp3, GATA3, and RORC was measured using the QuantiGene 2.0.
RESULTS: Results showed that Th17-type and Th0-type molecules' mRNAs, when compared with results obtained from tissue controls, were increased in biopsies of erosive lesions, whereas Th2-type molecules' mRNAs were increased in reticular lesions. When the CD4+ T-cell clones, derived from oral lichen planus tissues and tissue controls, were analyzed, a higher prevalence of Th17 (confirmed by an increased CD161 expression) and Th0 CD4+ T clones was found in erosive lesions, whereas a prevalence of Th2 clones was observed in reticular lesions.
CONCLUSIONS: Our data suggest that Th17, Th0, and Th2 cells, respectively, may have a role in the pathogenesis of erosive and reticular oral lichen planus.
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Keywords: Th17; lymphocytes; mucosa; oral; autoimmunity; lichen planus

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease that mainly shows reticular and erosive lesions (Scully *et al*, 1998; Baccaglini *et al*, 2013). OLP tends to be a chronic ailment showing along the years fluctuations in clinical signs and symptoms (Kaplan *et al*, 2012). The clinical variations of OLP reflect, microscopically, an elaborate morphological spectrum characterized by a combination of features such as orthokeratosis, parakeratosis, epithelial lamina atrophy, lymphocytic exocytosis, basal layer destruction, presence of Civatte bodies, and focal or multifocal or band-like lymphocytic infiltration in the upper lamina propria (Scully *et al*, 1998). In OLP, main pathogenetic mechanism consists in T-helper cell activation by a still unknown antigen, although other nonspecific mechanisms have been described (Kilpi, 1988; Sugerman *et al*, 2000; Roopashree *et al*, 2012). Mechanistic pathways include activation of Langerhans cells and/or keratinocytes, which present the antigen to CD4+ T cells. These cells secrete IFN-γ, a Th1-type cytokine, which upon activating CD8+ cytotoxic T cells may trigger basal keratinocyte apoptosis (Sugerman *et al*, 2000; Khan *et al*, 2003; Roopashree *et al*, 2012).

Beyond the traditional Th1 and Th2 cells, a new set called Th17 cells has been recently identified. These cells produce IL-17 A, IL-17 F, IL-6, TNF-α, GM-CSF, IL-21, IL-26, and IL-22 (Harrington *et al*, 2005). Th17 cells have an important role in the protection against extracellular bacteria and fungi, in inflammation, and in the development of autoimmune diseases (Cua *et al*, 2003; Murphy *et al*, 2003; Dubin and Kolls, 2008). A subset (named Th17/Th1 cells) of Th17 cells were found to also produce IFN-γ, and recent studies have reported that both Th17 and Th17/Th1 cells exhibit plasticity toward Th1 cells in response to IL-12 (Annunziato *et al*, 2007).

Little is known about the role of Th17 cells and associated cytokines in the pathogenesis of OLP. Few recent studies have investigated the role of serum IL-17 and Th1 and Th17 in OLP. Shaker and Hassan (2012) tried to measure IL-17A in the serum of patients with OLP using ELISA and concluded that IL-17 levels were higher in affected patients as compared to controls (mean ± s.d.: 19.76 ± 4.31 pg ml^{-1} *vs* 5.26 ± 1.45 pg ml^{-1}). However, 20% of the patients with OLP had viral hepatitis,

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and 64% were previously treated with topical and systemic corticosteroids, which downregulate T-cell responses. It is important to stress that the presence of IL-17 in the serum is not per se a definitive proof that Th17 cells are involved in OLP pathogenesis; in addition, ELISA tests are usually not sensible enough to be applied for this kind of investigation. More importantly, to make a reliable conclusion on their potential pathogenic role, Th17 cells have to be investigated directly in OLP lesions. Xie *et al* (2012) investigated the role of Th1 and Th17 cells in both lesions and serum of patients with OLP. Determining only the expression of IFN-γ and IL-17, these authors concluded that Th17 cells, as well as Th1 cells, were present in OLP lesions and that the number of peripheral blood Th17 cells in patients with erosive OLP was elevated when compared with that in patients with reticular OLP. However, it is important to underline that CD4+ T cells, which produce IFN-γ, are not by force Th1 cells; instead, if they produce Th2-type cytokines (as IL-4, IL-5, and IL-13) together with IFN-γ, they are Th0 cells and not Th1 cells.

Therefore, the aim of this study was to explore the potential pathogenic role and the proportions of Th17 subsets (Th17, Th17/Th1) and Th1, Th0 and Th2 cells present in lesional tissues of patients affected by reticular and erosive OLP, comparing the results with those of normal oral tissues (controls) obtained from the same group of patients with OLP.

Material and methods

Study population

The study group included 14 patients with OLP observed at our Center for the Study of Oral Diseases, Florence, Italy. Selected patients were only those with a definite diagnosis of OLP that never have been treated for their condition. Patients previously treated with corticosteroids or other immunosuppressive drugs were excluded. Also, patients affected by OLP plus other concomitant autoimmune disorders and patients taking immunosuppressive drugs for systemic or neoplastic diseases were excluded from the study. All 14 patients of the study group were from Tuscany (Italy), which epidemiologically is considered a low-prevalence geographical area for hepatitis C; thus, our institutional policy is not to routinely screen patients with OLP for HCV infection. Our patients reported no positive history of hepatitis C, and none of them belonged to risk groups (i.e., drug abuser or other categories) or reported prior blood transfusion. None of them had abnormal serum levels of liver enzymes (ALT and AST) (see Baccaglini *et al*, 2013). Patients with overt hepatitis C were excluded from the study. Diagnostic clinical criteria of OLP included bilateral white striae on oral mucosa accompanied, when present, by other clinical patterns such as erosive/ulcerative, atrophic, and plaque-like lesions. In all cases, the clinical diagnosis of OLP was confirmed by histopathological examination demonstrating features such as orthokeratosis and/or parakeratosis on the surface of the epithelium, atrophic rete ridges, destruction of the basal layer, and presence of a lymphocytic band-like infiltration at the interface between the epithelial lamina and lamina propria (Scully *et al*, 1998). Cases with histopathological evidence of epithelial dysplasia were excluded from the study. For each patient with OLP, two additional 3-mm punch biopsies were taken from both lesional tissues and clinically normal vestibular mucosa for the biological studies. In cases of reticular OLP, the 3-mm punch biopsy was taken from the white striae, while in cases of erosive OLP, the biopsy was taken from the ulcer margins. All biopsied samples were deep enough to contain both the epithelial lamina and subepithelial soft tissues. Biopsies of the healthy tissue controls were taken from clinically normal vestibular mucosa at least 3–4 cm away from the lesional areas. For each patient affected by OLP, healthy mucosa adjacent to lesional areas was chosen as tissue control to minimize the potential bias linked to the influence of oral flora on T-cell functional activity. A written consent was obtained from all participants in agreement with a protocol approved by the Human Ethical Committee of our University Hospital and in full accordance with the Declaration of Helsinki.

Quantization of mRNA for cytokines in OLP lesions and healthy tissue controls

mRNA expression for interleukin (IL)-17F, IL-17A, monocyte chemotactic protein-1 (MCP-1), IL-13, IL-2, IL-10, IL-1β, RANTES (CCL5), IL-4, IL-12B, IL-8, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-1α, IL-18, transforming growth factor (TGF)-β1, IL-23R, IL-7, IL-15, IL-6, MIG, interferon gamma-induced protein 10 (IP-10), CXCL10, lymphotoxin beta (LTB), vascular endothelial growth factor (VEGF), IL-5, IL-27, IL-23A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene for normalization of the results), peptidyl-prolyl cis–trans isomerase B (PPIB) (housekeeping gene for normalization), forkhead box P3 (Foxp3), GATA-binding protein 3 (GATA3), RAR-related orphan receptor C (RORC) was measured using the QuantiGene 2.0 (Panomics, Fremont, CA, USA) according to the manufacturer's instructions.

Generation of CD4+ T-cell clones (TCCs) from oral mucosal biopsies

We obtained two biopsies of erosive OLP, two of reticular OLP, and four control biopsies from healthy oral vestibular mucosa adjacent to the oral lesions (two for erosive OLP and two for reticular OLP). All specimens for this kind of evaluation were obtained from four additional patients with OLP. The tissues were cultured for 6 days in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol, 10% FCS (Hyclone Laboratories, Logan, UT, USA), and low levels of recombinant human IL-2 (20 U ml^{-1}) (Eurocetus, Milano, Italy) to culture only lesional activated T-cell blasts. TCCs were generated as previously described (Piccinni *et al*, 1998). Briefly, T-cell blasts were seeded under limiting dilution conditions (0.3 cell per well) in 96 round-bottomed microwell plates containing 10^5 irradiated (9000 rad) allogeneic PBMCs (as feeder cells) and PHA (1% vol/vol) in a final volume of 0.2 ml complete medium supplemented with IL-2 (50 U ml^{-1}) and 10% FCS. Growing microcultures were then supplemented, at weekly intervals, with IL-2 (50 U ml^{-1}) and

10^5 feeder cells. CD4 and CD8 distributions of T-cell clones were assessed by flow cytometer analysis (Becton Dickinson, Mountain View, CA, USA). To induce cytokine production, 10^6 T blasts from each clone were cultured in the presence of PMA (20 ng ml^{-1}) plus anti-CD3 mAb (100 ng ml^{-1}). After 24 h, culture supernatants were collected and stored at −70°C until used.

Determination of cytokine concentrations in TCC supernatants with bead-based multiplex immunoassays
The quantitative determination of IL-1beta, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IFN-alpha, TNF-alpha, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1-alpha, and MIP-1-beta was performed using a bead-based multiplex immunoassay and a Bioplex 200 system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Lédée *et al*, 2008).

Quantization of mRNA for cytokine and transcription factors in CD4+ TCC and CD161 expression in Th17-type CD4+ TCC derived from OLP lesions
T blasts were suspended in lysis solution at a concentration of 250 000 cells ml^{-1} and shaken at 65°C for 30 min. The lysates were stored at −80°C. mRNA expression for IL-17F, IL-17A, and ROR γT in CD4+ TCC from OLP lesions was measured using the QuantiGene 2.0 bead-based multiplex assay (Panomics) as described above. The Th17-type TCCs derived from OLP lesions were analyzed for CD161 surface expression with a flow cytometer, after staining with anti-CD3-pacific blue, CD4-PE/Cy7, and CD161-PE-conjugated mAbs (Becton Dickinson).

Statistics
Statistical analysis was performed using SPSS software (SPSS Inc, Evanston, IL, USA). All comparisons were performed by Wilcoxon test. T-helper subpopulation percentages were analyzed by chi-square test. A P value of < 0.05 was accepted as statistically significant.

Results

Patient's clinical features
The clinical findings of the 14 patients with OLP are summarized in Table 1. Five patients were men and nine women; median age was 54 years (range: 29–85). Eight patients were affected by reticular OLP and six by erosive OLP.

Table 1 Patient's clinical data

Case	*Age*	*Sex*	*Location*	*Clinical form*
1	30	M	Tongue	Reticular
2	38	F	Tongue, buccal mucosa	Reticular
3	72	M	Tongue, buccal mucosa	Reticular
4	58	F	Tongue, buccal mucosa, gingiva	Reticular
5	82	F	Buccal mucosa, gingiva	Reticular
6	69	F	Buccal mucosa, gingiva	Reticular
7	29	M	Buccal mucosa, tongue	Reticular
8	40	F	Tongue, buccal mucosa, gingiva	Reticular
9	39	F	Tongue, buccal mucosa	Erosive
10	50	F	Gingiva, buccal mucosa	Erosive
11	58	F	Tongue, buccal mucosa	Erosive
12	78	F	Tongue, buccal mucosa	Erosive
13	85	M	Buccal mucosa, gingiva	Erosive
14	34	M	Buccal mucosa, tongue, gingiva	Erosive

Th17- and Th0-type molecules are increased in erosive OLP, whereas Th2-type molecules predominate in reticular OLP
In reticular OLP, mRNA levels for IL-17 A, IL-17 F, IL-23 R, RORC (Th17-type molecules), and IFN-γ together with IL-4 and IL-5 (Th0-type cytokines) were not statistically different from Th17- and Th0-type molecules' mRNA levels in tissue controls except for GATA3 and IL-13 mRNA levels (Th2-type molecules), which appeared increased in reticular OLP when compared with tissue controls (Figure 1). In erosive OLP, mRNA levels for Th17 and Th0 molecules (IL-4, IL-5, IL-13, GATA3, and IFN-γ) were statistically higher than those in tissue controls (Figure 1). These results indicate that Th17 and Th0 cytokines may have a pathogenetic role in erosive OLP and Th2 cytokines in reticular OLP. No differences in mRNA expression for MCP-1, IL-2, IL-10, RANTES, IL-12B, IL-8, TNF-alpha, IL-1 alpha, IL-18, TGF-beta1, IL-7, IL-15, IL-6, MIG, IP-10, LTB, VEGF, IL-27, and Foxp3 were found in reticular and erosive OLP when compared with their respective tissue controls (data not shown). The levels of mRNA for the cytokines expressed in the tissue controls of both erosive and reticular OLP were not statistically different (Figure 1).

TCCs generated from erosive OLP produced higher levels of Th17- and Th0-type cytokines than those generated from healthy mucosa
To prove that the cytokines found were produced by CD4+ T cells and not by other oral mucosal cells, we generated CD4+ TCC from reticular and erosive OLP and from tissue controls. A total of 374 CD3+ CD4+ TCCs were generated from OLP-infiltrating T cells. We obtained 36 CD4+ TCCs from two biopsies of reticular OLP, 112 from two biopsies of the respective tissue controls, 132 from two biopsies of erosive OLP, and 94 from two biopsies of the respective tissue controls. T-cell cloning is a long and expensive technique usually used to confirm previous data obtained with another less expensive and faster technique (QuantiGene 2.0). Higher levels of IL-17 A (P = 0.001), IL-6 (P = 0.01), TNF-α (P = 0.006), GM-CSF (P = 0.005), IL-5 (P = 0.02), and IFN-γ (P = 0.01) were produced by CD4+ TCCs generated from erosive OLP in comparison with those produced by TCCs generated from tissue controls (Figure 2); instead, there was no significant difference in the production of any cytokines by the CD4+ TCCs generated from both reticular OLP and tissue controls. These results confirmed that CD4+ T cells, producing both Th0- (IL-5 and IFN-γ) and Th17-type cytokines (IL-17A, IL-6, TNF-α, GM-CSF), seem to have a critical role in the development of erosive OLP. As it was explained above, healthy oral mucosa, adjacent to OLP lesions, was chosen as tissue control to minimize the potential bias linked to the influence of oral flora on T-cell functional activity.

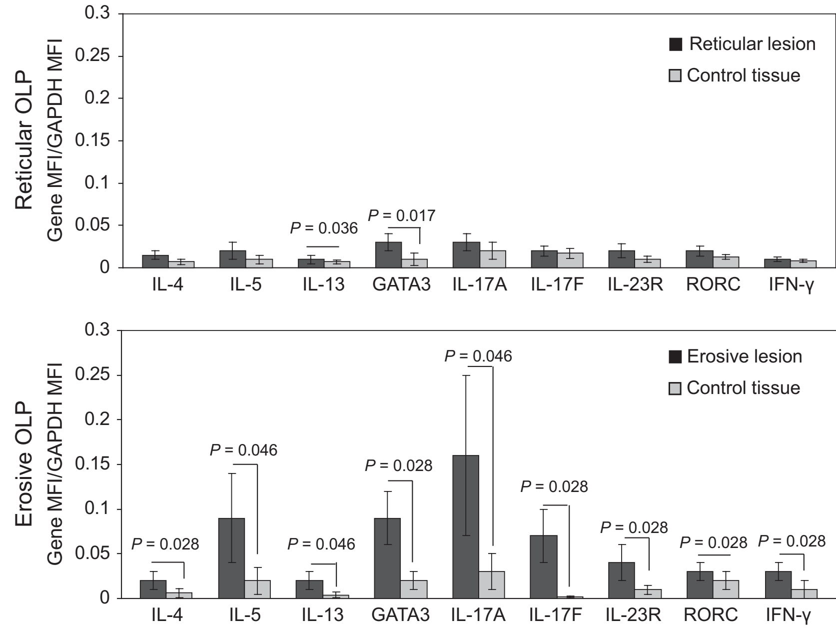

Figure 1 Increase in mRNA for Th17- and Th1-type molecules in erosive lesions compared with healthy mucosa. mRNA expression for IL-17 A, IL-17 F, IL-23 R, RORC, IL-4, IL-5, IL-13, GATA3, and IFN-γ in oral mucosal biopsies with erosive lesions (n = 6), reticular lesions (n = 8), and oral mucosal biopsy from the healthy oral vestibular mucosa adjacent to the oral erosive and reticular lesion (six controls for erosive lesions and eight controls for reticular lesions) (n = 14) obtained from patients suffering from oral lichen planus (OLP) has been quantified by a bead-based multiplex assay (QuantiGene 2.0)

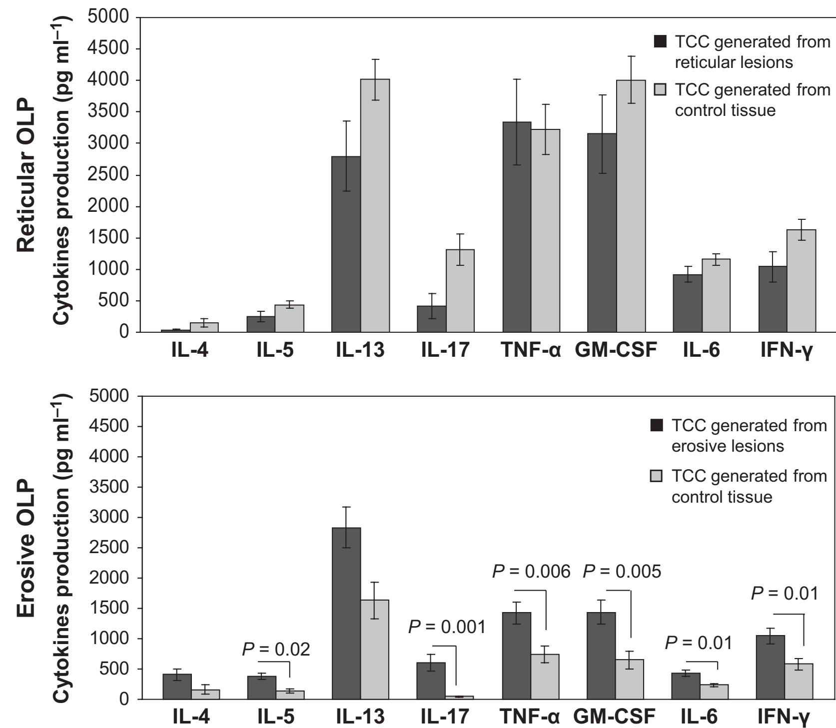

Figure 2 Increased Th17-type and Th0-type cytokines produced by CD4+ T-cell clones derived from erosive lesions. A total of 374 CD3+ CD4+ T-cell clones were generated from infiltrating T cells of oral mucosal biopsies with erosive lesions (n = 2) and reticular lesions (n = 2), and oral mucosal biopsy from the healthy oral vestibular mucosa adjacent to the oral erosive (n = 2) and adjacent to the reticular lesion (n = 2) of patients suffering from oral lichen planus (OLP). The levels of IL-17 A, IL-6, TNF-α, GM-CSF, IL-5, and IFN-γ) were measured in the supernatants of all the CD4+ T-cell clones obtained with a multiplex bead-based assay (Bio-Rad)

The proportion of Th17 plus Th0 cells is higher in erosive OLP, while Th2 cells are more prevalent in reticular OLP
We calculated the percentage of Th17-, Th0-, Th2-, and Th1-type CD4+ TCCs from the total number of CD4+ TCCs derived from biopsies of erosive and reticular OLP and from tissue controls. No statistical difference in the percentage of Th17, Th1, Th2, and Th0 was found analyzing separately each of the two patients with erosive lesions and each of the two patients with reticular lesions. Th17 clones that also expressed mRNA for IL-17A, IL-17F, and RORC (data not shown) were 84 of 364 (23%), Th2 clones 126 of 364 (34.6%), Th0 TCCs 144 of 364 (39,5%), and Th1 clones 10 of 364 (2,7%).

The percentage of Th17 cells was significantly higher in erosive lesions (35%) compared with tissue controls (4%) (P = 0.001) (Figure 3a). By contrast, there was no significant difference in the percentage of Th17 CD4+ TCCs generated from reticular OLP (4%) and their tissue controls (14%). There was also no significant difference in the percentage of Th1 TCCs (producing IFN-γ) generated from

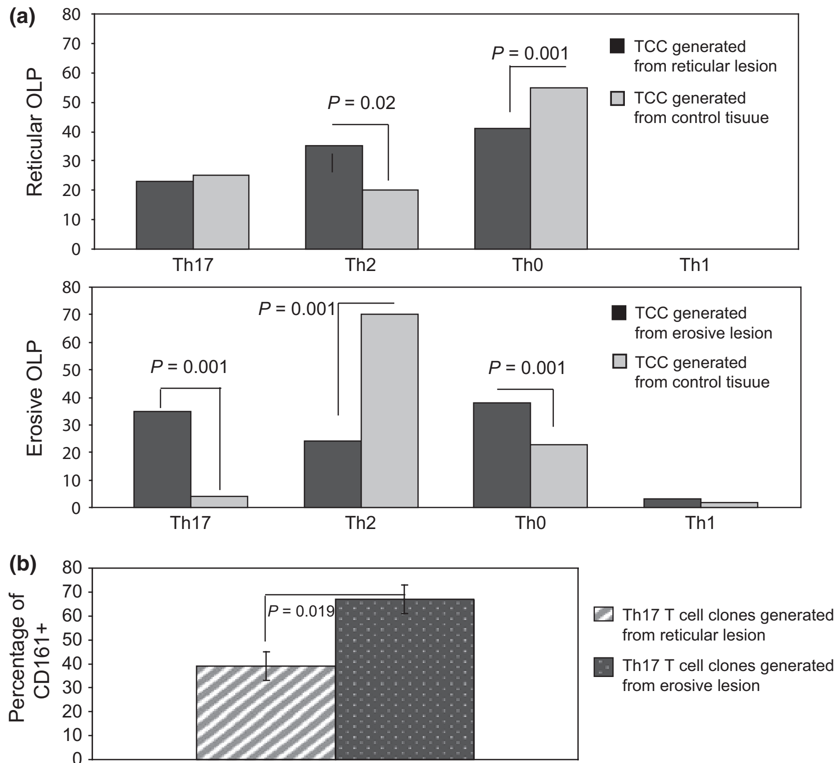

Figure 3 Percentage of Th17, Th0, and CD161+ cells and Th2 cells increased, respectively, in erosive and in reticular lesions. The percentage of Th17-, Th2-, Th0-, and Th1-type CD4+ T-cell clones obtained from the total number of CD4+ T-cell clones (n = 374) derived from infiltrating T cells of oral mucosa with erosive lesions (n = 2), reticular lesions (n = 2), and healthy oral vestibular mucosa adjacent to the erosive (n = 2) and reticular lesions (n = 2) of the same patients suffering from oral lichen planus (OLP) were calculated. We confirmed with QuantiGene 2.0 that all the IL17-A-producing CD4+ T-cell clones obtained, selected on the basis of the cytokines produced in the supernatants, expressed m RNA for IL-17A, IL-17F, and RORC (Figure 3a). The percentage of CD4+ T cells expressing CD161 were evaluated with flow cytometry in Th17-type CD4+ T-cell clones derived from OLP erosive lesions (n = 20) in comparison with the percentage of Th17-type CD4+ T-cell clones derived from OLP reticular lesions (n = 20) (Figure 3b)

erosive OLP (3%) and their tissue controls (2%), whereas there was absolutely no Th1 TCC generated from reticular OLP and their tissue controls. However, the percentage of Th0 TCCs (producing IFN-γ together with IL-4, IL-5, or IL-13) was significantly higher in erosive OLP (38%) compared with their tissue controls (23%) (P = 0.001), whereas the percentage of Th0 TCCs was significantly decreased in reticular OLP (41%) compared with their tissue controls (55%) (P = 0.001). By contrast, the percentage of Th2 TCCs (producing IL-4, IL-5, or IL-13) was significantly higher in reticular OLP (35%) compared with tissue controls (20%) (P = 0.02), whereas the percentage of Th2 TCCs was significantly decreased in erosive OLP (24%) compared with their tissue controls (70%) (P = 0.001). The percentage of pure Th17 TCCs (that produce IL-17A, not associated with other cytokines) was increased in erosive OLP (35%) compared with the percentage of their tissue controls (4%) (P = 0.025), whereas no increase in the pure Th17 TCCs number in reticular OLP was observed in relation to their tissue controls (data not shown). Therefore, the percentage of Th17 and Th0 cells appears higher in erosive OLP, whereas the percentage of Th2 cells is higher in reticular OLP. Th1 cells seem to have no significant role in both erosive and reticular OLP. No Th17/Th1 cells were present in any of the OLP lesions or in tissue controls.

We investigated the percentage of CD4+ cells expressing CD161 in Th17 TCC derived from erosive OLP (n = 20) in comparison with the percentage of Th17 TCC derived from reticular OLP (n = 20) (Figure 3b). We found a significant increased expression of CD161 in IL-17A-producing TCC derived from erosive OLP (67%) compared with CD161 expression of IL-17A-producing TCC (39%) derived from reticular OLP (P = 0.019). As the human memory Th17 cells are contained within the CD161+ fraction of circulating and tissue-infiltrating CD4+ T cells (Cosmi *et al*, 2008; Kleinschek *et al*, 2009) , these data confirmed the critical role that Th17 may have in the pathogenesis of erosive OLP.

Discussion

Our results indicate that Th17 and Th0 cells may have a pathogenetic role in erosive OLP as well as Th2 cells in reticular OLP. Similar to Khan *et al* (2003) and Xie *et al* (2012), we found that T cells in OLP lesions produce IFN-γ. However, our results are not in agreement with Khan's findings, who reported that infiltrating T cells in OLP predominantly are Th1 cells without a T cell–IL-4 production (Khan *et al*, 2003). Xie *et al*, 2012 did not evaluate IL-4 and did not prove that infiltrating T cells are Th1 or Th0 cells. We found that the IFN-γ production and mRNA expression in erosive OLP are quite exclusively associated with Th2-type cytokines, and this reflects the Th0 profile.

We speculate that in erosive OLP, Th17 cytokines (which have a role in inflammation) and Th0-type cytokines (which include IFN-γ production that can activate CD8+ cells) may be responsible for the more evident oral mucosal damage, whereas the decreased number of Th0 cells and the increased Th2 cells (which are not pathogenic!) can explain the less evident epithelial damage associated with reticular OLP. Although in previous studies on OLP (Sugerman *et al*, 2000), Th2-type cytokines were not detected, the presence of IL-4 in peripheral blood and saliva of patients with OLP has been recently reported (Kalogerakou *et al*, 2008; Liu *et al*, 2009).

The plasticity of Th17 cells toward Th1 cells is linked to the activity of IL-12 or to the prolonged exposure of Th17 cells to IL-23 (Lee *et al*, 2009; Cosmi *et al*, 2011). Thus, naïve CD4+ CD161+ T cells, precursors of Th17 cells (Cosmi *et al*, 2008), can differentiate into Th17, Th17/Th1, and finally, Th1 cells, in response to environmental IL-12. However, the respective role of Th17, Th17/Th1, and Th1 cells in the pathogenesis of chronic inflammatory disorders is still debated. It is notorious that Th17 cells can promote inflammation, but it has been observed that they can induce autoimmune diseases only after their conversion into Th1 cells (Doodes *et al*, 2008; Bending *et al*, 2009; Martin-Orozco *et al*, 2009). In our study, the very minimal presence of Th1 cells (only 3%) and the prevalence of Th17 cells (35% of the total generated TCCs), expressing high levels of CD161, suggest that a complete shifting of Th17 toward Th1 cells does not occur in erosive OLP. The prevalence of Th17 and the defect of Th1 cells, but also the defect of Th17/Th1 cells (in particular in both erosive and reticular OLP), may be related to the lack in the microenvironment of local factors necessary for the differentiation of Th17 cells into Th1 cells. However, we observed that mRNA for IL-12 and IL-23, measured by QuantiGene 2.0, was increased significantly in both reticular and erosive OLP when compared with tissue controls (data not shown). Thus, the lack of differentiation of Th17 toward Th1 cells in OLP tissues cannot be only explained by the lack of IL-12 or IL-23 in the microenvironment in which the infiltrating T cells reside. In OLP, local factors that can block the Th17 plasticity toward Th1 or other external factors necessary for driving Th17 plasticity toward Th1, but not present in the local mucosal milieu, have to be investigated.

It is of utmost importance to discuss the reasons beyond our choice of selecting, for each patient affected by OLP, the clinically normal vestibular mucosa as tissue control. It is universally known that patients with OLP show variable involvement of the oral mucosa; some areas show typical lesions, while others are devoid of lesions. Histologically, lesional areas present different grades of the well-known features that characterize OLP. By contrast, biopsies taken from normal-appearing mucosa do not show any of the previous aspects but, in general, reveal only few scattered mononuclear cells mainly in the upper lamina propria (data not shown). The significance of these mononuclear cells is that they represent stable resident memory T cells, which physiologically colonize both normal oral mucosa and skin. These memory T cells provide long-lasting, local, and rapid responses to pathogen re-exposure, but can also contribute to inflammatory and autoimmune diseases (see the review paper by Clark, 2010). These considerations and the basic histopathological observation discussed above led us to choose the normal vestibular mucosa as tissue control. The biopsy site was selected at least 3–4 cm away from the OLP lesional areas; therefore, the possibility that portions of OLP were incorporated into the healthy mucosa was minimized. Although peripheral blood has been used as control by some research groups, in our opinion, blood does not represent an appropriate control against the oral mucosa. The main reason is that the immune cell composition of peripheral blood is quite diverse from the immune cells of oral mucosa. Even though oral tissues and peripheral blood are obtained from the same patient with OLP, the results obtained from the mucosal lesions cannot be comparable with those obtained from peripheral blood mainly because blood cells exhibit functional activities completely different from those of the oral mucosa. Those diversities are due to a different array of cytokines, associated with the two tissues, which influence the differentiation of CD4+ T cell toward Th cell subsets. In conclusion, the type of tissue microenvironment, either normal or pathologically altered, may have a pivotal role in influencing the results of studies that want to analyze the functional activity of T cells present in pathological tissues such as OLP. By contrast, we think that peripheral blood from normal donors could be the appropriate control for peripheral blood of patients affected by OLP. Furthermore, we have chosen not to use as control the oral mucosa of healthy donors not affected by OLP, because in our experimental conditions, we were able to eliminate the differences in the T-cell functional responses exhibited by two groups of different individuals (patients and normal donors), and therefore, we were able to highlight only the T-cell functional differences related to the presence of OLP in the same patient (data not shown). Also, it could be argued that the diversity of oral flora pathogens present in the mouth of two different individuals may influence the T-cell functional activity. Our choice of sampling OLP lesions and healthy tissue (control) in the same patient also minimized this potential bias. Although production of some cytokines (IL-13, TNF-α, GM-CSF, IL-6) derived from TCCs generated from tissue controls of patients with reticular OLP appeared higher than TCCs generated from tissue controls of patients with erosive OLP (Figure 2), other cytokines (and in particular IL-17) produced by TCCs derived from tissue controls of patients with reticular OLP statistically did not differ from those produced by TCCs derived from tissue controls of patients with erosive OLP. A TCC, which consists of more than 10^6 cells, is derived from a single T cell that grows in culture with a recombinant T-cell growth factor (rIL-2) and feeder cells (irradiated fresh allogeneic peripheral blood mononuclear cells from different healthy donors) (Piccinni *et al*, 1998), which support the T-cell culture for 2 months. For a certain tissue specimen, the feeder cells used for all the cultures are the same for both lesional tissues and the tissue controls, because the two T-cell clonings are performed together in the same conditions. This is also a reason why we can compare the results obtained between TCC derived from lesional OLP tissues and TCC derived from tissue controls. Obviously, for the subsequent analysis of an additional specimen, the feeder cells, used for the cultures of both lesional and control tissues, will be diverse because the biopsies are obtained after a certain period of time, then the healthy donors of feeder cells would be different. The fact that some feeder cells can be more or less supportive for the TCC may explain the differences in the results obtained from the tissue controls of patients with reticular OLP when matched against the results gathered from tissue controls of patients with erosive OLP. It is of relevance to stress that our results were obtained comparing TCC generated from both lesional OLP tissue and tissue controls that were cultured in the same conditions and with the same feeder cells (Figure 2). According to our interpretation, there is no evidence of statistical differences between

the levels of mRNA for all the cytokines expressed in tissue controls of patients with erosive OLP and tissue controls of patients with reticular OLP (Figure 1).

Our findings have a great relevance also in view of new treatments for OLP. Recent findings suggested that the neutralization of IL-17 or a humanized monoclonal antibody that inhibits both IL-12 and IL-23 could be a potential novel approach for the treatment of immune-mediated disease (Gottlieb *et al*, 2009; Genovese *et al*, 2010).

In conclusion, our investigation supports evidence that Th17 together with Th0 and Th2 cells, respectively, may play a pivotal role in the pathogenesis of OLP. However, we are aware that more future work is necessary to clarify the definitive pathways through these T-cell subsets that participate to the pathogenic mechanisms of OLP.

Conflict of interest

The authors state no conflict of interest.

Author contributions

All authors gave contribution to the study. M-P. Piccinni, L. Lombardelli, F. Logiodice, O. Kullolli, R. Biagiotti, and M. G. Giudizi mainly contributed to the laboratory investigations and statistical analysis. G. Ficarra and D. Tesi involved in the clinical and histological investigations and handling of patient's data. M-P. Piccinni, G. Ficarra, and D. Tesi contributed to the conception, drafting, and writing the final version of the manuscript. S. Romagnani and E. Maggi involved in reviewing the final version of the manuscript.

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